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Technology refinement for production of Serratiopeptidase from *Serratia marcescens*

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BIOTECHNOLOGY

By

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Ph.D. Thesis Synopsis

Title of the Thesis	**:**	Technology refinement for production of Serratiopeptidase from *Serratia marcescens*	
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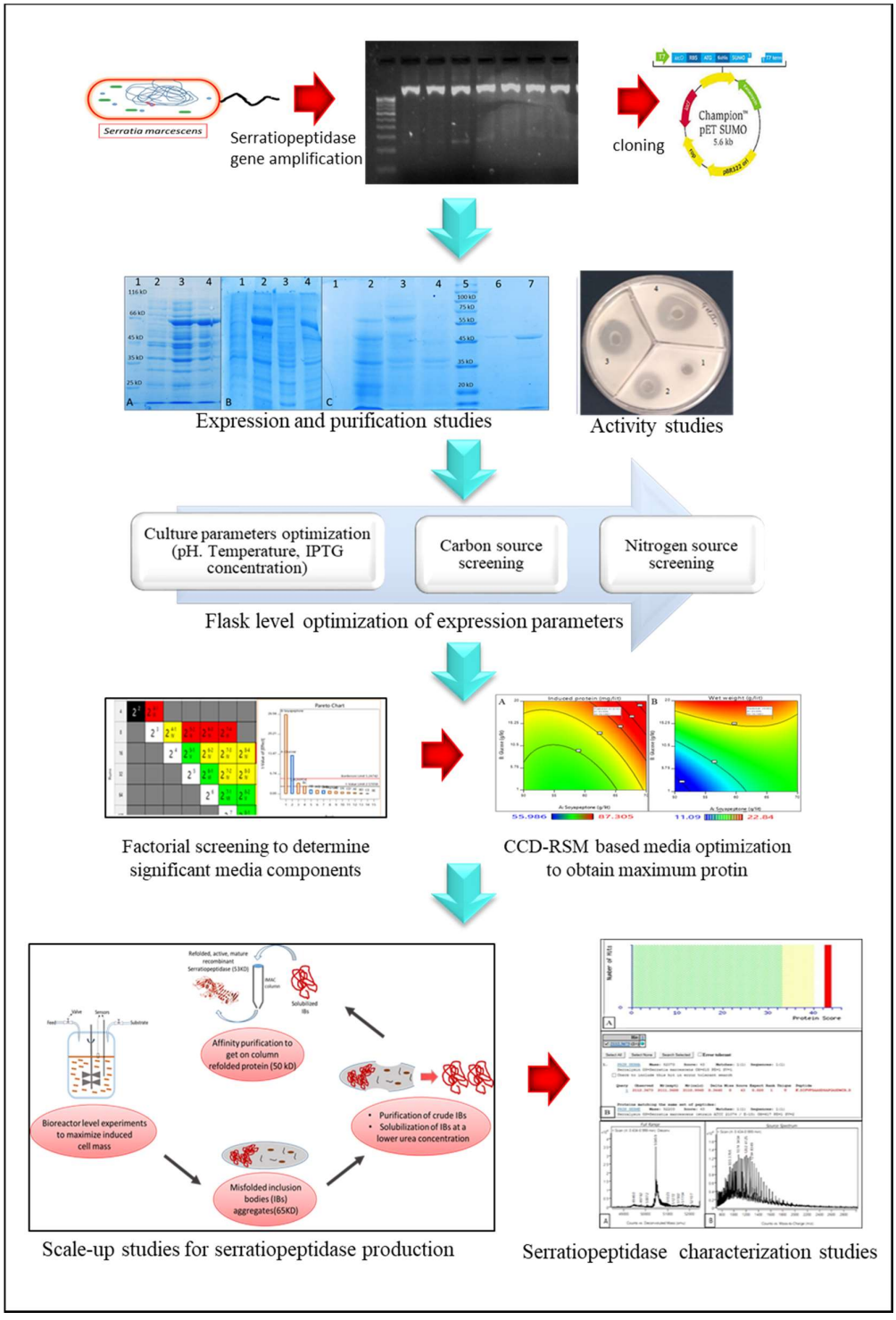
1. Abstract

Microbial enzymes increasingly find applications as therapeutics due to their targeted activity and minimal side effects. Serratiopeptidase, a miracle enzyme, has already proven its therapeutic potential in many studies. Serratiopeptidase is an extracellular zinc-containing metalloprotease molecular weight of about 50kD and was first reported to be produced by *Serratia marcescens*. It has shown therapeutic (anti-inflammatory, anti-fibrinolytic and analgesic) as well as industrial applications (detergents, food processing, leather, paper and brewing etc.). The evolution of *Serratia marcescens* as an opportunistic pathogen associated with various infections has led researchers to think of and develop an alternate strategy for its industrial production. The study presents successful cloning, expression and purification of active serratiopeptidase, using *Escherichia coli* BL21 [DE3] and pET SUMO vector followed by optimization of synthetic media and culture conditions for enhanced serratiopeptidase production. Initial optimization of physical parameters was done by screening different carbon and nitrogen sources. As shown by the factorial screening experiment, the significant media components for serratiopeptidase production were subjected to Response Surface Methodology (RSM) based optimization. The optimized media yielded 86 mg L^{-1} of biologically active refolded serratiopeptidase from 20 g L^{-1} wet weight of induced pellet as predicted by the equation. This was followed by developing a cost-effective, bioreactor-level production process comprising the fed-batch fermentation to produce recombinant serratiopeptidase protein expressed as a fusion construct. Optimizing bioreactor parameters obtained a high yield of cell mass and protein. The downstream solubilization and purification processes were also optimized to achieve maximum yield of pure, active serratiopeptidase protein. A final yield of 2.5 ± 0.764 g L^{-1} of protein was obtained, having 8382±291 U mg^{-1} of specific caseinolytic activity. Additionally, a novel, unexpected self-proteolytic activity of the enzyme cleaves the N-terminal 6× His-SUMO fusion tag along with the enzyme propeptide, thus yielding a mature serratiopeptidase, was also found. The success of the application of a statistical model for designing an optimized media for enhanced serratiopeptidase production also suggests a new insight for the scale-up of serratiopeptidase towards therapeutic and industrial applications.

Graphical Abstract


Serratia marcescens
Serratiopeptidase gene amplification
cloning
Champion™ pET SUMO 5.6 kb
Expression and purification studies
Activity studies
Culture parameters optimization (pH. Temperature, IPTG concentration)
Carbon source screening
Nitrogen source screening
Flask level optimization of expression parameters
Factorial screening to determine significant media components
CCD-RSM based media optimization to obtain maximum protin
Affinity purification to get on column refolded protein (50 kD)
Bioreactor level experiments to maximize induced cell mass
Purification of crude IBs
Solubilization of IBs at a lower urea concentration
Misfolded inclusion bodies (IBs) aggregates(65KD)
Scale-up studies for serratiopeptidase production
Serratiopeptidase characterization studies

2. Introduction

Enzymes as biocatalysts are very efficient, and specific, and accelerate the reaction by more than a millionfold compared to an uncatalyzed reaction (Wolfenden and Snider 2001). Microbes are a source of various enzymes that have various industrial and therapeutic applications as biocatalysts. The advent of metagenomics, genome mining and exploration studies of extremophiles have aided in exploring microbial enzyme diversity. Due to their easy susceptibility to genetic manipulation, microbes are also used for recombinant expression of enzymes with high yield and minimal downstream purification process (Rosano, German L and Morales, Enrique S and Ceccarelli 2019).

Serratiopeptidase (also known as serralysin/serrapeptase/serratia protease) is a proteolytic enzyme initially reported to be produced by *Serratia marcescens*. The enzyme also finds therapeutic application as an anti-inflammatory, anti-edemic, analgesic and fibrinolytic agent (Jadhav et al. 2020). Positive effects of serratiopeptidase have been reported in different medical procedures, including orthopaedics, ophthalmology, surgery, gynaecology, otorhinolaryngology, pulmonology and dentistry (Santhosh Kumar 2018). Studies on rat models have also shown that in Alzheimer's disease, serratiopeptidase may also be considered as a neuroprotective agent since it alleviates the neuroinflammation and apoptosis associated with the disease (Fadl et al. 2013). Moreover, due to its mucolytic property, serratiopeptidase has also been proposed to act as a potent drug in the integrative management of COVID-19 (Sharma et al. 2021). The exact mode of action of serratiopeptidase as a therapeutic is unclear; however, the proposed mode of action of Serratiopeptidase is

1. Regulating the recruitment of PMMs and other lymphocytes at the site of inflammation (Mouneshkumar Chappi et al. 2015)
2. Reducing capillary permeability induced by histamine, bradykinin, and serotonin (Tiwari 2017)
3. Solubilizing non-living tissues such as mucous, plaques and blood clots by proteolysis and facilitating the absorption of decomposed products through blood and lymphatics (Nakamura et al. 2003)

The current demand for serratiopeptidase in pharmaceuticals and industries is met by using a wild or mutated form of its natural strain *S. marcescens*. The pathogenic nature of the producer bacteria and the threats associated with the disposal of the bulk biomass after fermentation demand a need to look into alternative production strategies using recombinant DNA technology. Reports for recombinant production of serratiopeptidase are available, but many of them could not produce an active enzyme due to unstable expression plasmids, formation of inclusion bodies or production of an inactive enzyme (Ethiraj and Gopinath 2017). Successful expression and refolding of recombinant serratiopeptidase from inclusion bodies in different *E.*

coli vectors have been reported in some recent studies (Srivastava et al. 2019). However, high-yield and cost-effective production of recombinant serratiopeptidase by bioreactor-level fermentation has not yet been reported.

Considering the significance of the serratiopeptidase enzyme as mentioned above, as well as for the industrial-level production of serratiopeptidase, formulation of optimum media must be done to maximize the yield and reduce the cost of production. As per our knowledge, reports for statistical media optimization for industrial level recombinant serratiopeptidase production are unavailable. This study presents our work to develop technology for recombinant serratiopeptidase production. Cloning of the serratiopeptidase gene was done in *E. coli* BL21. After confirmation of expression, statistical media optimization using a factorial experiment to eliminate noncontributing factors for serratiopeptidase production was done, followed by Central composite design – Response surface methodology (CCD-RSM) to optimize significant factors for maximizing the production of recombinant serratiopeptidase to obtain pure, refolded active serratiopeptidase.

After successful clone selection and screening for recombinant protein production at the flask level, bioreactor systems are used for large-scale production to fulfil the demands of industrial biopharmaceuticals using high throughput process development techniques (Tripathi and Shrivastava 2019). Even though a significant amount of literature is available for fed-batch production of recombinant proteins in *E. coli* (Lee 1996; Krause et al. 2016; EL Moslamy 2019), optimum conditions for culture growth, product formation and accumulation need to be defined for each expression system due to variation in promoters, sequence of expressed recombinant protein, and its effect on host physiology. Earlier reports (Yee 1992; Lee 1996) have also stated that even though parameters like plasmid stability, copy number, and expressed product stability and solubility are defined by the genes of host and vectors, the culture conditions and media composition also affect the same. Hence each system needs optimization in terms of culture conditions and media constituents to achieve maximum product accumulation.

Thus after flask-level studies, optimization studies for high-density fed-batch fermentation process were done for recombinant serratiopeptidase production. Using the statistically optimized media (OM) as basal media, both batch and fed-batch fermentations were performed to maximise the yield in terms of cell mass and recombinant protein product. Optimization for efficient lysis and solubilization of inclusion bodies from the bulk pellet, followed by on-column refolding, was done to obtain active serratiopeptidase from inclusion bodies. MALDI MS/MS for identification further characterized the purified protein, and intact mass analysis was done by LCMS to determine the autocleavage site upon refolding.

3. Objectives of the study

- Strain selection and identification of the gene for Serratiopeptidase.
- Strain development for over-expression of Serratiopeptidase.
- Compare activity of improved strain with natural strain.
- Optimization of fermentation conditions and downstream processing for overproduction of serratiopeptidase.

4. Materials and Methods

Chemicals, bacterial host strains and plasmids

Luria-Bertani (LB) medium was used to grow *E. coli* in the initial transformation and cloning experiments. The expression host used for the transformation of the recombinant plasmid was BL21 [DE3] F^- ompThsdSB (rB $^-$ mB $^-$) gal dcm (DE3). The Champion pET SUMO vector harboring the kanamycin resistance gene from Invitrogen Lifesciences was used for cloning and expressing serratiopeptidase.

Gene cloning and protein expression studies

Serratia marcescens BAB 3285 (Accession no. MN945437) culture from the Bank A Bug repository (WDCM No.1058) GBRC Gandhinagar, India, was used as a source of serratiopeptidase gene. The complete coding sequence for the serratiopeptidase gene was obtained from NCBI GeneBank. The forward primer 5′ ATGCAATCTACTAAAAAGGC 3′ and reverse primer 5′ TTACACGATAAAGTCCG 3′ were designed using Gene Runner software (www.generunner.net) and used for serratiopeptidase gene amplification. The amplified gene was cloned in the Champion pET SUMO vector by TA cloning using the manufacturer's protocol (Champion pET SUMO Protein Expression System). The vector has a small ubiquitin-like modifier (SUMO) to allow the expression, purification, and generation of native proteins in *E. coli*. The transformation of the cloned vector was done in *E. coli* BL21 strain by the chemical method ($CaCl_2$ transformation). PCR confirmed that clones have the serratiopeptidase gene in the desired orientation. Clones were sequenced, and the obtained sequence was deposited in NCBI GenBank.

A single colony of clones confirmed with the correct orientation of the serratiopeptidase gene was inoculated in LB broth. The overnight-grown culture was used as inoculum for expression studies. The culture was incubated in a shaker set at 37 °C with 200 rpm. Selection pressure was maintained using 50 µg/mL of Kanamycin. Induction for recombinant protein expression was done by adding 1mM of IPTG into the medium when the cell density in terms of OD_{600} reached 0.6. The overexpression of the recombinant serratiopeptidase was checked by SDS–PAGE. The gel was stained using Coomassie brilliant blue to visualize the protein bands. It was further analyzed for protein expression.

After confirmation of protein expression with expected mass, experiments were performed to optimise other parameters like IPTG concentration, pH and temperature. The overexpressed protein was checked for its solubility. The protein in inclusion bodies was purified through affinity chromatography by on-column refolding and checked for activity.

Media optimization studies

The major components of synthetic media to be used for serratiopeptidase production consisted of carbon source (10 gL^{-1}), nitrogen source (40 gL^{-1}), dipotassium hydrogen orthophosphate (13 gL^{-1}), magnesium sulfate (1.2 gL^{-1}) and trace metal solution (25 mlL^{-1}). Experiments were initially conducted to screen for the best carbon source (Dextrin, Glucose, Glycerol, Maltodextrin, Maltose and starch) and nitrogen source (Ammonium phosphate, Corn steep liquor and soya peptone) for recombinant serratiopeptidase production. Results were recorded in terms of maximum induced protein production and colony-forming unit (CFUs/ml) of culture. For defining the optimum media for recombinant serratiopeptidase production, statistical optimization experiments were performed to reduce the efforts and time required in formulating the same. Design Expert 11 software (Stat-Ease Inc., Minneapolis, MN) was used for experiments designing and statistical analysis of the recorded responses. The significant media components responsible for maximum serratiopeptidase production were determined using the regular two-level factorial designs. The factorial design helped to determine the effect of a factor over the range which was used to experiment on the desired response. The selected media components were screened at 2 levels to determine factors affecting the serratiopeptidase production by the clone in 21 experimental trials and having 5 center points. The factors showing the p-value of <0.05 were used for further optimization using response surface methodology (RSM).

Central Composite Design was used for the optimization of three significant factors viz. dipotassium hydrogen phosphate, Glucose, and soya peptone as identified by full factorial design. These variables were studied at 5 different levels ($-\alpha$, -1, 0, 1, $+\alpha$). Concentrations of components were given in terms of alpha values. The alpha level was kept at 1.68 and a set of 20 experiments as given by the software were performed in triplicates.

Bioreactor level experiments

Luria Bertani (LB) broth was used as seed culture media; the Optimized media (OM) was used as basal media for the bioreactor cultivation experiments. The OM contained glucose (16gL^{-1}), Soyapeptone (66gL^{-1}), dipotassium hydrogen phosphate (12.7gL^{-1}), magnesium sulfate (1.2gL^{-1}) and trace metal solution (25mlL^{-1}). Feed media for high-density cell mass contained carbon (glycerol and/or glucose), nitrogen (Yeast extract and peptone), trace metal solution and magnesium sulphate. All the bioreactor experiments were performed in a 5L New Brunswick BioFlo 115 reactor (Eppendorf, Germany). In addition, fed-batch experiments were carried out to optimize conditions yielding maximum cell mass and induced protein.

Optimization of protein purification and specific activity studies

Cell lysis and inclusion bodies solubilization conditions were optimized by subjecting the inclusion bodies to solubilization under different conditions (Varying urea concentrations, varying pH, use of detergents etc.). The solubilized protein was subjected to column purification using the Biologic LP system and eluted in the native elution buffer using on-column protein refolding. A gelatin hydrolysis assay was performed for qualitative estimation of the purified enzyme.

The caseinolytic assay was used to determine the specific activity of the purified serratiopeptidase. Casein was subjected to hydrolysis by the purified serratiopeptidase, and the specific activity was estimated in terms of the μmoles of tyrosine released after hydrolysis during the given incubation period.

Characterization of purified serratiopeptidase

The purified protein was subjected to MALDI MS/MS analysis for identification of the purified protein. Intact mass analysis of the auto-cleaved purified protein was done by LCMS analysis.

5. Results and Discussion

Cloning and expression studies

The serratiopeptidase gene amplification was done using the designed primers, and full-length coding DNA sequences (CDS) of 1464 base pairs (bp) were amplified. The clone having the gene in proper orientation was confirmed by using vector forward and gene reverse primer. Vector-specific primers were used to obtain the complete ORF of the cloned gene. The gene sequence was deposited in NCBI GenBank (Accession no. MN967013).

The clone containing the correct orientation of the serratiopeptidase gene with maximum expression was selected for further optimization studies. The minimum IPTG concentration giving maximum induction at flask level was found to be 0.6 mM, as confirmed by densitometric analysis. In our study, the optimum pH for recombinant serratiopeptidase production was 7, as confirmed by the SDS PAGE of the induced pellet at different pH. The maximum induction was after 16 hrs of induction at 30 ^{0}C. Henceforth, the above optimum conditions were used for media optimization.

The recombinant serratiopeptidase was found to be expressed as inclusion bodies. The enzyme was initially purified using a denaturation protocol using urea to obtain the enzyme eluted in a buffer containing urea and having a size of 63kD and SUMO protein. Later, on-column folding was optimized to obtain native enzyme (Molecular weight 50kD) without His tag and SUMO fragment. The purified recombinant serratiopeptidase showed proteolytic activity, as seen on Gelatin agar plates. The folding protocol yielded 46.24±0.61 mg L^{-1} of purified refolded serratiopeptidase as estimated by Bradford assay. The inclusion bodies were solubilized, and on-

column folding was optimized to get native serratiopeptidase showing caseinolytic and proteolytic activity on Gelatin agar plates.

Media optimization studies

Of the different carbon and nitrogen sources screened, media containing glucose as a carbon source and soyapeptone as a nitrogen source gave the best results in terms of CFUs ml^{-1} and induced protein as estimated by densitometric analysis in SDS PAGE of the induced pellet of different treatments.

The basal media used consisted of 5 main components: carbon, nitrogen, Dipotassium hydrogen phosphate, Magnesium sulfate, and trace metal solution (TMS). Glucose is usually the preferred carbon source because it is readily uptaken and metabolized by most bacteria. However, when using the T7 expression system, glucose acts as a catabolite repressor of the operon (Wong et al. 1997). Hence, a lower glucose concentration was used for obtaining the initial log phase early due to the fast growth and multiplication of cells in the presence of glucose. It is assumed that most of the glucose is consumed during induction. Dipotassium hydrogen phosphate here serves as a buffering agent to maintain the media's pH and is a source of Phosphorus and Potassium for culture. Soyapeptone is a soluble end product obtained by the enzymatic hydrolysis of defatted soybean meal. It is a rich source of essential amino acids, carbohydrates and vitamins for obtaining luxuriant growth. Nagel et al., 1999, reported that the culture showed a shorter lag phase when their media was supplemented with soya peptone, i.e. log phase was attained early compared to supplementation of other nitrogen sources and vitamins. In this study, using the recombinant strain, it was also observed that soya peptone was a better nitrogen source of the screened sources for serratiopeptidase production. Magnesium sulfate served as a source of magnesium ions required for DNA replication and trace metal solution as a source of calcium and zinc required for the serratiopeptidase enzyme and was a source of other micro elements required for the culture growth.

The variation in concentration of expressed recombinant serratiopeptidase in the factorial design reflected the need to optimize the media composition for serratiopeptidase production. Glucose, Soyapeptone and Dipotassium hydrogen phosphate were found to be significant media components, as seen through the p values of the screened media components. These were further subjected to CCD-RSM, and the responses were recorded in terms of the wet weight of the pellet and induced protein concentration as estimated by densitometric analysis. The model F-values implied that the model was significant for both responses. The coefficient of correlation (R^2) was found to be greater than 0.97, thus implying that at least 97% of experimental data obtained from the CCD optimization experiment was compatible with the data predicted by the model. The optimized media formulation was defined to contain glucose ($16gL^{-1}$), Soyapeptone ($66gL^{-}$

^{1}), dipotassium hydrogen phosphate (12.7gL^{-1}), magnesium sulfate (1.2gL^{-1}) and trace metal solution (25mlL^{-1}).

Bioreactor level experiments

Both batch and fed-batch fermentations were carried out to check the protein yield at the bioreactor level. The cell mass in optimized media was more than twice in LB broth in terms of absorbance as well as dry cell mass. The flask-level production experiments yielded recombinant serratiopeptidase up to 43mgL^{-1} in LB broth and 86mgL^{-1} in OM from 9 and 20 gL^{-1} of wet cell mass, respectively. There was a significant increase in the yield regarding cell mass and, ultimately, the induced protein at the bioreactor level.

Feed media was supplemented when nutrients were exhausted in fed-batch mode, as indicated by an increase in DO concentration. Two different feeding strategies were attempted to enhance cell mass and induced protein concentration by using different carbon sources in the feed media. In the first strategy, only glycerol was used as a carbon source for feeding. In the second set of experiments, initial feeding was done using glucose-containing feed media (7% w/v of basal media volume) at the rate of 100mlh^{-1}. Upon exhaustion of nutrients, second feed supplementation containing glycerol (10% v/v of basal media volume) was started, and induction was also given simultaneously. A higher induced cell mass was obtained when only glycerol was used as a carbon source. Although glucose is a simpler carbon source readily used by most microbes, a higher cell mass and corresponding induced protein yield was obtained in this study using only glycerol in feed media as compared to glucose followed by glycerol. Similar results have been reported by Korz et al., 1995, where a higher biomass was obtained in glycerol as feed media as compared to glucose, mainly due to the higher concentration of glycerol in the feed medium since glucose has limited solubility (Korz et al. 1995).

The DO concentration in the batches went below 5% during the high-density fermentation experiments. Protein expression is also affected by DO concentration. In order to cope with the reduced oxygen concentration, experiments were performed using the instrument's maximum agitation rate (1200rpm). The relative yield of cell mass and induced protein was found to be decreased at the higher agitation rate which may be due to mechanical stress induced in the cells, leading to reduced expression.

A lower temperature is also known to improve protein expression and folding. However, switching to a lower temperature (20 ^{o}C) after induction in bioreactor batches did not improve the induced protein per unit cell mass and also yielded a lower amount of cell mass as compared to batch incubated at 30 ^{o}C.

Optimization of protein purification

Cell lysis was done by sonication, followed by nuclease treatment. Next, the solubilization procedure was optimized using 2M urea-containing buffer followed by overnight incubation at -20 ºC to enhance solubilization. Finally, protein purification was done using on-column refolding. Briefly, after binding the protein of solubilized protein on the affinity column, urea was gradually removed from the wash buffer, followed by elution of recombinant serratiopeptidase native elution buffer containing Imidazole. The size of purified serratiopeptidase in the native elution buffer was around 50kD compared to the induced fusion protein having a size of ~ 65kD. This showed that the protein was getting auto-cleaved upon refolding. Further characterization of the protein was thus done to confirm its identity and cleavage site.

Characterization of purified serratiopeptidase

The identity of the pure protein was confirmed by peptide mass fingerprinting, wherein the pure protein was trypsinized and subjected to mass spectrometry. The fragmentation pattern matched with serratiopeptidase protein with a significant score. Further, the identity was confirmed by MS/MS analysis, wherein the peptide sequence of one of the peaks matched with serratiopeptidase protein.

The peak data obtained after trypsinization of recombinant serratiopeptidase showed that the peak having m/z of 2112.3 was further subjected to MS/MS. The peak data obtained from the same was further uploaded on the MASCOT database, and the protein was identified as serratiopeptidase (UNIPROT ID - P07268 (PRZN_SERME)) with a significant score of 43. The MS/MS peak data of the protein confirmed it to be serratiopeptidase with the help of the MASCOT database. The sequence coverage data of the recombinant serratiopeptidase obtained by PMF was 23%.

In order to identify the cleavage site in the purified serratiopeptidase, the intact mass of the protein was determined by LC-MS. The intact mass of the purified recombinant serratiopeptidase was estimated to be 50.459 kD, which matched the mass of mature serratiopeptidase protein without the propeptide (50.457 kD) as analyzed through the sequence data. The auto-cleavage of recombinant serratiopeptidase could be attributed to the inherent proteolytic activity of the serratiopeptidase enzyme

6. Conclusions

Serratiopeptidase has shown promising results as a therapeutic agent in various studies. However, threats associated with using the native strain have led researchers to think about alternate production strategies. As mentioned earlier, recombinant serratiopeptidase was found to be expressed as inclusion bodies and could not be purified in an active form. After initial

flask-level studies, the cautious optimization of the fed-batch procedure at the various cultivation steps yielded pure, refolded, and active serratiopeptidase with the maximum yield per liter of media and maximum specific activity, to the best of our knowledge. Although the protein yield is higher than in the earlier studies, there is still further scope for improvement. As mentioned earlier, the DO level, which reached below 5% during the cultivation, can be increased by aeration using air enriched with oxygen, and increasing the feed supplements with a more extended batch period might still enhance the cell mass and induce a higher protein yield per batch to obtain higher cell densities. Furthermore, due to its proteolytic activity, the self-cleavage of recombinant serratiopeptidase upon refolding is being reported for the first time, which demands further study.

7. References

1. EL Moslamy SH (2019) Application of Fed-Batch Fermentation Modes for Industrial Bioprocess Development of Microbial Behaviour OPEN ACCESS
2. Ethiraj S, Gopinath S (2017) Production, purification, characterization, immobilization, and application of Serrapeptase: a review. Front. Biol. (Beijing). 12:333–348
3. Fadl NN, Ahmed HH, Booles HF, Sayed AH (2013) Serrapeptase and nattokinase intervention for relieving Alzheimer's disease pathophysiology in rat model. https://doi.org/10.1177/0960327112467040
4. Garrido F, Estrela S, Alves C, et al (2011) Refolding and characterization of methionine adenosyltransferase from *Euglena gracilis*. Protein Expr Purif 79:128–136
5. Han K, Lim HC, Hong J (1992) Acetic acid formation in *Escherichia coli* fermentation. Biotechnol Bioeng 39:663–671
6. Jadhav SB, Shah N, Rathi A, et al (2020) Serratiopeptidase: Insights into the therapeutic applications. Biotechnol. Reports 28
7. Kante RK, Vemula S, Somavarapu S, et al (2018) Optimized upstream and downstream process conditions for the improved production of recombinant human asparaginase (rhASP) from *Escherichia coli* and its characterization. Biologicals 56:45–53. https://doi.org/10.1016/j.biologicals.2018.10.002
8. Korz' DJ, Rinas U, Hellmuth K, et al (1995) Simple fed-batch technique for high cell density cultivation of *Escherichia coli*
9. Krause M, Neubauer A, Neubauer P (2016) The fed-batch principle for the molecular biology lab: Controlled nutrient diets in ready-made media improve production of recombinant proteins in Escherichia coli. Microb. Cell Fact. 15
10. Lee SY (1996) High cell-density culture of *Escherichia coli*. Trends Biotechnol 14:98–105

11. Mouneshkumar Chappi D, Suresh K V, Patil MR, et al (2015) Comparison of clinical efficacy of methylprednisolone and serratiopeptidase for reduction of postoperative sequelae after lower third molar surgery. J Clin Exp Dent 7:e197
12. Nageswara S, Singam R, Guntuku G (2016) Design of a low cost fermentation medium for the production of Serratiopeptidase enzyme by a novel *Streptomyces sp*. World J Pharm Pharm Sci 5:829–842
13. Nakamura S, Hashimoto Y, Mikami M, et al (2003) Effect of the proteolytic enzyme serrapeptase in patients with chronic airway disease. Respirology 8:316–320
14. Rosano, German L and Morales, Enrique S and Ceccarelli EA (2019) Rosano, 2019.pdf. Protein Sci 28:1412–1422. https://doi.org/https://doi.org/10.1002/pro.3668
15. Rosano GL, Ceccarelli EA (2014) Recombinant protein expression in *Escherichia coli* : advances and challenges. 5:1–17. https://doi.org/10.3389/fmicb.2014.00172
16. Santhosh Kumar M (2018) The emerging role of serratiopeptidase in oral surgery : literature update. 11:
17. Sharma C, Jha NK, Meeran MFN, et al (2021) Serratiopeptidase, A Serine Protease Anti-Inflammatory, Fibrinolytic, and Mucolytic Drug, Can Be a Useful Adjuvant for Management in COVID-19. Front Pharmacol 12:. https://doi.org/10.3389/fphar.2021.603997
18. Singh A, Upadhyay V, Upadhyay AK, et al (2015) Protein recovery from inclusion bodies of *Escherichia coli* using mild solubilization process. Microb Cell Fact 14:. https://doi.org/10.1186/s12934-015-0222-8
19. Srivastava V, Mishra S, Chaudhuri TK (2019) Enhanced production of recombinant serratiopeptidase in *Escherichia coli* and its characterization as a potential biosimilar to native biotherapeutic counterpart. Microb Cell Fact 18:. https://doi.org/10.1186/s12934-019-1267-x
20. Tiwari M (2017) The role of serratiopeptidase in the resolution of inflammation. Asian J. Pharm. Sci. 12:209–215
21. Tripathi NK, Shrivastava A (2019) Recent Developments in Bioprocessing of Recombinant Proteins : Expression Hosts and Process Development. 7:. https://doi.org/10.3389/fbioe.2019.00420
22. Wolfenden R, Snider MJ (2001) The depth of chemical time and the power of enzymes as catalysts. Acc Chem Res 34:938–945
23. Wong P, Gladney S, Keasling JD (1997) Mathematical Model of the lac Operon : Inducer Exclusion , Catabolite Repression , and Diauxic Growth on Glucose and Lactose. 132–143
24. Yee L (1992) Recombinant Protein Expression in High Cell Density Fed-Batch Cultures of *Escherichia coli*. Nat Biotechnol 10:1550–1556

8. Conference attended

- **Doshi P,** Bhargava P, Pathak C, Joshi C and Joshi M, Poster presentation on " Cloning and Purification Studies of Serratiopeptidase in *E. coli* ", at international conference MOLMED – 2019 held from 25th – 27th September 2019, at Changa, Gujarat.
- Participated in national level conference Gujarat Vigyan Sammelan (GVS 2022) held from 30th January 2022 to 5th February 2022.

9. Publications

- Doshi, P., Bhargava, P., Singh, V., Pathak, C., Joshi, C. and Joshi, M., 2020. *Escherichia coli* strain engineering for enhanced production of serratiopeptidase for therapeutic applications. International Journal of Biological Macromolecules, 160, pp.1050-1060. (Research paper)
- Doshi, P., Dantroliya, S., Modi, A., Shukla, A., Patel, D., Joshi, C. and Joshi, M., 2022. Enhanced Production Process of Recombinant Mature Serratiopeptidase in *Escherichia coli* Using Fed-Batch Culture by Self-Proteolytic Activity of Fusion Protein. *Fermentation*, *8*(7), p.307. (Research paper)
- Doshi, P., Shri, M., Bhargava, P., Joshi, C.G. and Joshi, M., 2020. Microbial Production of Industrial Proteins and Enzymes Using Metabolic Engineering. In *Engineering of Microbial Biosynthetic Pathways* (pp. 189-204). Springer, Singapore. (Book chapter)

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